8-Isoprostane Enzyme Immunoassay Kit

Catalog No. 133-16465

96 Well Kit

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Sapphire Bioscience Pty Ltd • ABN 60 055 311 236 • Email: Sales@sapphirebioscience.com

Description

Sapphire's 8-isoprostane (8-*iso* $PGF_{2\alpha}$) EIA kit is a competitive immunoassay for the quantitative determination of 8-*iso* $PGF_{2\alpha}$ in biological samples. This assay is based on the competition between 8-*iso* $PGF_{2\alpha}$ and a 8-*iso* $PGF_{2\alpha}$-alkaline phosphatase tracer for a limited amount of 8-*iso* $PGF_{2\alpha}$-specific antiserum. Because of the competition between 8-*iso* $PGF_{2\alpha}$ in the sample and 8-*iso* $PGF_{2\alpha}$ tracer for the 8-*iso* $PGF_{2\alpha}$ antiserum, the signal obtained with the assay will be inversely proportional to the amount of 8-*iso* $PGF_{2\alpha}$ in each sample. This equilibration is performed in the wells of a 96-well plate pre-coated with mouse monoclonal anti-rabbit IgG, which binds all of the 8-*iso* $PGF_{2\alpha}$ antiserum added to the well. After the incubation step, the plate is washed, and a solution of *para*-nitrophenyl phosphate (*p*NPP), a substrate for alkaline phosphatase, is added. The product of this enzymatic reaction has a distinct yellow color which absorbs at 412 nm, allowing quantification of 8-*iso* $PGF_{2\alpha}$ in each sample.

Introduction

8-Isoprostane (8-*iso* $PGF_{2\alpha}$) is a member of a large family of $PGF_{2\alpha}$ isomers that are formed by random free radical-mediated peroxidation of esterified arachidonic acid within cellular membranes. 8-*iso* $PGF_{2\alpha}$ was discovered while performing routine mass spectral analysis for 11β-$PGF_{2\alpha}$ in plasma samples as a species that accumulated non-enzymatically as a result of improper storage.[1,2] Of the 64 theoretical $PGF_{2\alpha}$ isomers that are possible from arachidonic acid, 8-*iso* $PGF_{2\alpha}$ is the most well studied and most commonly measured. Currently, two primary systems of nomenclature are used to describe the isoprostane family members, which are based on 4 regioisomers formed from arachidonic acid.[3,4] 8-*iso* $PGF_{2\alpha}$ is synonymous with the names $iPF_{2\alpha}$-III and 15-$F_{2\alpha}$-isoprostane (15-$F_{2\alpha}$-*iso*P).

8-*iso* $PGF_{2\alpha}$ is hydrolyzed from membrane phospholipids, presumably by PLA_2, circulates in the plasma and is then excreted in the urine. Plasma from healthy volunteers contain modest amounts of 8-*iso* $PGF_{2\alpha}$ (40-100 pg/ml) and normal human urinary levels range from 10-50 ng/mmol creatinine.[5,6] Levels of 8-*iso* $PGF_{2\alpha}$ are elevated in a variety of clinical and pathological settings that are associated with increased oxidative stress, including smoking, diabetes, atherosclerosis, and several neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.[7-9]

Precautions

1. Please read all instructions carefully before beginning this assay.
2. Some reagents contain azide, which may react with lead or copper plumbing. If disposing of reagents in the sanitary sewer, please flush with a large volume of water to prevent azide build-up.
3. This kit is for research use only. It is not intended for human or diagnostic use.

Materials Supplied

1.	8-Isoprostane Antiserum	1 vial	133-64653
2.	8-Isoprostane AP Tracer	1 vial	133-64652
3.	8-Isoprostane Standard	1 vial	133-64651
4.	Mouse Anti-rabbit IgG Coated Plate	1 plate	133-00008
5.	Tris Buffer Concentrate	2 vials	133-00003
6.	AP Wash Buffer Concentrate	1 vial	133-00004
7.	DEA Buffer Concentrate	1 vial	133-00001
8.	*p*NPP Tablets	5 tablets	133-00002
9.	Plate Cover	1 cover	133-00005

Storage

This kit should be stored at -20ºC and used before the expiration date printed on the box.

Materials Needed but Not Supplied

1. A source of Millipore water
2. Adjustable pipettors
3. Materials used for sample preparation
4. An orbital shaker
5. A plate reader capable of measuring absorbance between 405-420 nm

Sample Handling

This assay only measures the free (*i.e.* unesterified) form of 8-Isoprostane. However, in some samples such as plasma and tissues, 8-Isoprostane$_{\alpha}$ may be predominantly esterified in phospholipids. For measurement of total 8-Isoprostane in these sample types (*i.e.* free + esterified), base hydrolysis is required to release 8-Isoprostane from phospholipids prior to analysis. For more information, we refer you to references 10-12.

*[**Note**: Samples should be stored at -80°C in the presence of 0.005% BHT if unable to be assayed immediately following collection.]*

Materials needed for sample purification

1. 8-Isoprostane standard to use in determination of recovery
2. 1.0 M HCl, methanol, Millipore water, hexane, and ethyl acetate
3. 6 ml SPE C-18 cartridges

Purification Procedure

While the following purification protocol works well for many samples, it is important to be aware that it is not sufficient for the purification of 8-Isoprostane from all samples. For some matrices, it may be necessary to complement the following procedure with HPLC or TLC.

1. Split samples into two equal parts and place each sample into clean test tubes.
2. Spike one of these sets of samples with 8-Isoprostane.
3. Perform the following steps on all samples:
    a. Acidify the sample to pH <4.0 by the addition of 1.0 M HCl.
    b. Prepare a C-18 SPE cartridge by conditioning it first with 5 ml of methanol followed by 5 ml Millipore water.
    c. Apply the sample and allow to flow through the column.
    d. Rinse the column with 5 ml Millipore water, followed by 5 ml hexane. Allow the column to become dry following the hexane wash.
    e. Elute the 8-Isoprostane with 5 ml ethyl acetate containing 1% methanol. If unable to run the assay at this time, store the samples in the ethyl acetate/methanol at -80°C.
    f. Dry the sample under a stream of nitrogen. Reconstitute the sample in Tris Buffer and assay both the unspiked and spiked samples in the EIA.

Reagent Preparation

All diluted buffers should be stored at 4°C. When stored in this manner, they will be stable for approximately two months.

1. Tris Buffer

Dilute the contents of one vial of Tris Buffer Concentrate with 90 ml of Millipore water. It is common for the concentrated buffer to contain crystalline salts after thawing. It is important to rinse the vial to obtain any salts that may have precipitated.

2. Wash Buffer

Dilute the 5 ml vial of Wash Buffer to a final volume of 750 ml with Millipore water.

3. DEA Buffer

Dilute the 2.5 ml vial of DEA Buffer Concentrate to a final volume of 25 ml with Millipore water.

4. 8-Isoprostane Standard

*[**Note**: If assaying culture medium samples that have not been diluted with Tris Buffer, the culture medium rather than Tris Buffer should be used for dilution of the standard curve.]*

Equilibrate a pipet tip in ethanol, by repeatedly filling and expelling. Use the equilibrated pipet tip to transfer 100 µl of the 8-Isoprostane standard into a clean test tube. Dilute with 900 µl Millipore water. The concentration of this standard is 30 ng/ml. Label eight glass test tubes #1 - #8. Pipet 900 µl of Tris Buffer into tube #1, and 500 µl into tubes #2 - #8. Transfer 100 µl of the 30 ng/ml standard into tube #1 and vortex to mix. Transfer 250 µl from tube #1 to tube #2. Vortex to mix. Transfer 250 µl from tube #2 to tube #3. Vortex to mix. Continue this process for standard tubes #4 - #8. These diluted standards should be used within twenty-four hours.

5. **8-Isoprostane Alkaline Phosphatase Tracer**

Reconstitute the 8-Isoprostane Alkaline Phosphatase Tracer with 6 ml of Tris buffer. Vortex to mix.

Store this reconstituted tracer at 4ºC and use within four weeks.

6. **8-Isoprostane Antiserum**

Reconstitute the 8-Isoprostane Antiserum with 6 ml of Tris Buffer. Vortex to mix.

Store this reconstituted antiserum at 4ºC and use within four weeks.

Assay Procedure

*[**Note**: All reagents should be allowed to warm to room temperature before use.]*

1. **Tris Buffer**

Pipet 150 µl Tris Buffer into non-specific binding (NSB) wells, and 100 µl Tris Buffer into zero standard (B_0) wells. If tissue culture medium was used to dilute the standard curve, substitute 100 µl of this same medium for the Tris Buffer in the NSB and B_0 wells.

2. **8-Isoprostane Standard**

Pipet 100 µl of standards into the appropriate wells.

3. **Samples**

Pipet 100 µl of samples into the appropriate wells. Each sample should be assayed in duplicate or triplicate.

4. **8-Isoprostane Alkaline Phosphatase Tracer**

Pipet 50 µl of tracer into each well except the blank wells and total activity (TA) wells.

5. **8-Isoprostane Antiserum**

Pipet 50 µl of antiserum into each well except the blank wells, TA wells and NSB wells.

Well	Tris Buffer	Std/Sample	Tracer	Antiserum
Blank				
TA			5 µl (at development)	
NSB	150 µl		50 µl	
B_0	100 µl		50 µl	50 µl
Std/Sample		100 µl	50 µl	50 µl

Pipetting Summary

6. **Incubate the plate**

Cover each plate with a plate cover and incubate for two hours at room temperature on an orbital shaker.

7. **Prepare the *p*NPP Solution**

Dissolve 5 *p*NPP tablets in 25 ml DEA buffer (25 ml is sufficient to develop 100 wells). ***Note:*** *Reconstituted pNPP is not stable, so we recommend that you make only the amount that you need at any one time.*

8. **Wash the plate**

Empty the wells and rinse five times with wash buffer. After the final wash, firmly tap the inverted plate on a paper towel to remove any recalcitrant drops of buffer.

9. **Develop the plate**

Add 200 µl *p*NPP solution to each well including blank and TA wells. Add 5 µl of tracer to the TA wells. Cover the plate and allow to develop in the dark on an orbital shaker. This assay typically develops in approximately 60-90 minutes.

10. **Read the plate**

Wipe the bottom of the plate with a paper towel to remove any finger prints, smudges or dirt which may interfere with obtaining an accurate reading of absorbance. Remove the plate cover, and read the plate at a wavelength of between 405 and 420 nm.

Data Analysis

Most plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as $\%B/B_0$ versus log concentration of standard using either a 4-parameter logistic or a log-logit curve fit.

1. Prepare the Data

The following procedure is recommended to prepare the data prior to graphing (subtract the absorbance of the blank wells from all wells on the plate, if not already done).

a. Average the absorbance readings from the NSB wells.

b. Average the absorbance readings from the B_0 wells.

c. Subtract the average NSB from the average B_0. This is the corrected B_0.

$$\text{Corrected } B_0 = \text{Average } B_0 - \text{Average NSB}$$

d. Calculate the $\%B/B_0$ for each standard and sample. To do this, subtract the average NSB absorbance from the absorbance and divide by the corrected B_0 (from step c). Multiply by 100 to obtain $\%B/B_0$. Repeat for all wells.

$$\%B/B_0 = \left[\frac{\text{Absorbance - Average NSB}}{\text{Corrected } B_0}\right] \times 100$$

2. Plot the Standard Curve

Plot $\%B/B_0$ for all standards *versus* 8-isoprostane concentration using log (x) and linear (y) axes, and fit the data to a four parameter logistic equation. Alternatively, the data can be linearized using a logit transformation. *[**Note**: Do not use $\%B/B_0$ in this calculation.]*

$$\text{Logit } (B/B_0) = \ln [B/B_0/(1-B/B_0)]$$

Plot the data as logit(B/B_0) *versus* log concentration of standard and perform a linear regression fit.

3. Determine the Concentration of your Samples

Calculate the $\%B/B_0$ for each sample. Determine the concentration of each sample using the equations obtained from the analysis of the standard curve. Remember to account for any dilutions made to the sample prior to addition to the well. $\%B/B_0$ values of greater than 80% or less than 20% should be re-assayed as they generally fall outside of the linear range of the standard curve.

4. Correct for Recovery (if purification was performed)

Divide the concentration determined in step #3 by the recovery factor. Correct for any volume changes of the sample which may have occurred during purification.

$$\text{Recovery Factor} = \frac{\text{EIA value of spiked sample (pg/ml) - EIA value of unspiked sample (pg/ml)}}{\text{Concentration of spike (pg/ml)}}$$

Assay Performance Characteristics

1. Precision

Intra-assay precision was determined by measuring samples containing low, medium, and high concentrations of 8-isoprostane multiple times in the same assay (eight samples per plate on a total of five plates). Inter-assay precision was determined by measuring low, medium, and high concentrations of the samples eight times using different reagents.

	8-*iso* $PGF_{2\alpha}$ (pg/ml)	**Intra-assay %CV**	**Inter-assay %CV**
High	333.3	4.7	6.6
Medium	111.1	5.5	7.3
Low	37.0	8.1	12.6

2. Specificity

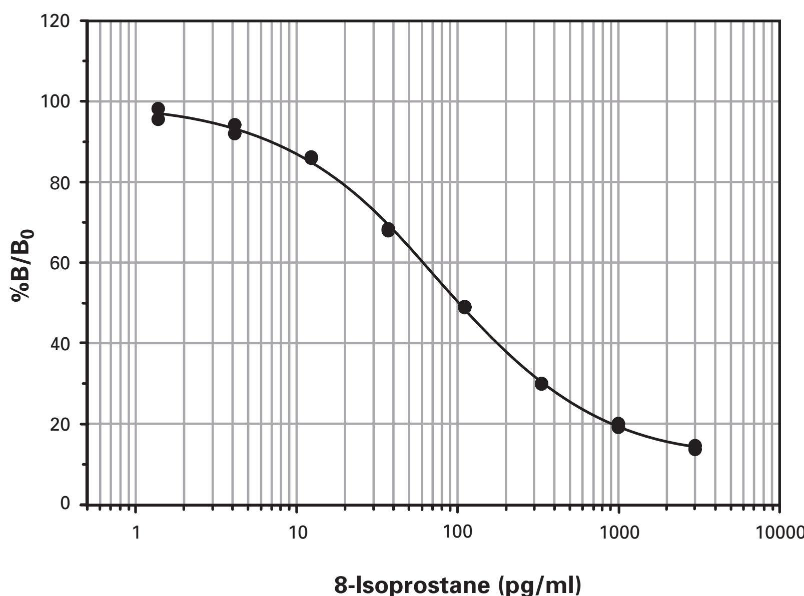
Analyte	Cross-Reactivity	Analyte	Cross-Reactivity
8-*iso* Prostaglandin $F_{2\alpha}$	100%	8-*iso* Prostaglandin $F_{1\beta}$	0.08%
8-*iso* Prostaglandin $F_{3\alpha}$	20.55%	Thromboxane B_2	0.08%
2,3-dinor-8-*iso* Prostaglandin $F_{2\alpha}$	4.00%	11-dehydro Thromboxane B_2	0.07%
8-*iso* Prostaglandin E_2	1.84%	11β-Prostaglandin $F_{2\alpha}$	0.03%
2,3-dinor-8-*iso* Prostaglandin $F_{1\alpha}$	1.70%	Prostaglandin E_2	0.02%
Prostaglandin $F_{1\alpha}$	0.71%	8-*iso*-15(R)-Prostaglandin $F_{2\alpha}$	0.02%
Prostaglandin $F_{3\alpha}$	0.66%	8,12-*epi* $iPF_{2\alpha}$-III	0.01%
Prostaglandin E_1	0.39%	$iPF_{2\alpha}$-VI	<0.01%
Prostaglandin D_2	0.16%	8,12-*epi* $iPF_{2\alpha}$-VI	<0.01%
6-keto Prostaglandin $F_{1\alpha}$	0.14%	13,14-dihydro-15-keto Prostaglandin $F_{2\alpha}$	<0.01%
Prostaglandin $F_{2\alpha}$	0.14%	Tetranor PGEM	<0.01%
2,3-dinor-6-keto Prostaglandin $F_{1\alpha}$	0.09%	Tetranor PGFM	<0.01%

3. Typical Results

The standard curve shown here is an example of data typically produced by this kit. Your results will vary from these, and it is therefore important that you run a standard curve each time you use the kit.

	Raw Data		Average	Corrected
Total Activity	728	709	718.5	
NSB	0	0	0	
B_0	633	652	654.3	654.3
	692	640		

Concentration Std (pg/ml)	Raw Data		Corrected		%B/B_0	
3,000	95	89	95	89	14.5	13.6
1,000	125	131	125	131	19.1	20.0
333.3	196	195	196	195	30.0	29.8
111.1	321	319	321	319	49.1	48.8
37.0	444	447	444	447	67.9	68.3
12.3	564	562	564	562	86.2	85.9
4.1	616	602	616	602	94.2	92.0
1.4	625	642	625	642	95.6	98.1



50% B/B_0 - 100 pg/ml
20% B/B_0 - 20 pg/ml

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